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TITLE: Therapeutic Strategies against Cyclin E1-Amplified Ovarian Cancers

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14. ABSTRACT Approximately 20% of high grade serous ovarian cancers harbor Cyclin E1 (CCNE1) amplification and are associated with poor outcome and inferior responsiveness to standard platinum chemotherapy. Given their intrinsic resistance to platinum, management of CCNE1-amplified ovarian cancers is challenging. In this research, we evaluate three novel strategies against CCNE1-amplified ovarian cancers that address different aspects of CCNE1 biology. In the first aim, based on our preliminary data, we hypothesize that HSP90-inhibitors may be effective against CCNE1-amplified ovarian tumors because they suppress HR, downregulate BRCA1, and downregulate CCNE1. In the second aim, based on our preliminary data and the fact that RB functions downstream of cyclin E, we hypothesize that inhibition of FOXM1 and RB interaction is an effective approach for targeting CCNE1-amplified ovarian tumors. Specifically, suppression of FOXM1/RB interaction will lead to enhancement of RB/E2F interaction and suppression of E2F-dependent oncogenic activity resulting in activity against CCNE1-amplified cells. In the third aim, we hypothesize that miR-1255b, miR-148b*, and miR-193b* may be effective against CCNE1-amplified ovarian tumors in combination with platinum and PARPis. Potential mechanisms for this effect include suppression of HR and downregulation of BRCA1, RAD51 and BRCA2 that are relevant for CCNE1-amplified ovarian tumors which are dependent on hyperactive HR and are sensitive to suppression of BRCA1.					
15. SUBJECT TERMS <i>Ovarian Cancer, CCNE1 amplification, Homologous Recombination, Platinum analogues, MicroRNAs, Heat shock protein 90 inhibitors, Forkhead box protein M1, Retinoblastoma</i>					
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1. INTRODUCTION:

Approximately 20% of high grade serous ovarian cancers harbor Cyclin E1 (CCNE1) amplification and are associated with poor outcome and inferior responsiveness to standard platinum chemotherapy. Given their intrinsic resistance to platinum, management of CCNE1-amplified ovarian cancers is challenging. In this research, we evaluate three novel strategies against CCNE1-amplified ovarian cancers that address different aspects of CCNE1 biology. In the first aim, based on our preliminary data, we hypothesize that HSP90-inhibitors may be effective against CCNE1-amplified ovarian tumors because they suppress HR, downregulate BRCA1, and downregulate CCNE1. In the second aim, based on our preliminary data and the fact that RB functions downstream of cyclin E, we hypothesize that inhibition of FOXM1 and RB interaction is an effective approach for targeting CCNE1-amplified ovarian tumors. Specifically, suppression of FOXM1/RB interaction will lead to enhancement of RB/E2F interaction and suppression of E2F-dependent oncogenic activity resulting in activity against CCNE1-amplified cells. In the third aim, we hypothesize that miR-1255b, miR-148b*, and miR-193b* may be effective against CCNE1-amplified ovarian tumors in combination with platinum and PARPis. Potential mechanisms for this effect include suppression of HR and downregulation of BRCA1, RAD51 and BRCA2 that are relevant for CCNE1-amplified ovarian tumors which are dependent on hyperactive HR and are sensitive to suppression of BRCA1.

2. KEYWORDS:

Provide a brief list of keywords (limit to 20 words).

Epithelial Ovarian Cancer, CCNE1 amplification, Homologous Recombination DNA Repair, Platinum analogues, MicroRNAs, Heat shock protein 90 (HSP90) inhibitors, Forkhead box protein M1 (FOXM1), Retinoblastoma (RB), Poly-ADP Ribose Polymerase Inhibitors (PARP-inhibitors)

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the USAMRAA Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals and objectives of the project?

The major goal for Aim 1 is to determine the activity of HSP90 inhibitors in CCNE1-amplified ovarian tumors.

The major goal for Aim 2 is to inhibit FOXM1 and RB interaction to suppress CCNE1-amplified ovarian tumors.

The major goal for Aim 3 is to determine the activity of certain miRNA mimics in combination with PARP-inhibitors or platinum against CCNE1-amplified ovarian tumors.

What was accomplished under these goals?

Since the starting of the award, substantial progress has been made toward achieving the goals as outlined in the application.

For AIM 1:

During the first year of the award we were able to achieve the following goals:

1) The HSP90-inhibitor 17-AAG has single agent activity against a number of CCNE1-amplified cell lines

As proposed in Aim 1, we evaluated the activity of HSP90-inhibitor 17-AAG in various CCNE1-amplified ovarian cancer lines (1), including OVCAR3, COV318 and in the OVCAR4 cell line which harbors CCNE1 overexpression. To that end, cells were plated at 1000 cells per well on a 96- well plate in sextuplicate and treated with 17-AAG at indicated concentrations on the next day. After 5 days, cell viability was quantified by Celltiter Glo.

As shown in Figure 1A, we noted significant activity of 17-AAG in OVCAR3 and OVCAR4 cell lines with IC50 of 0.07 and 0.08uM respectively. 17-AAG was also active in COV381 with an IC50 of 0.14uM, albeit less than OVCAR3 and OVCAR4. This is consistent with our hypothesis that HSP90 inhibitors may have good single agent activity against CCNE1 amplified and overexpressing lines.

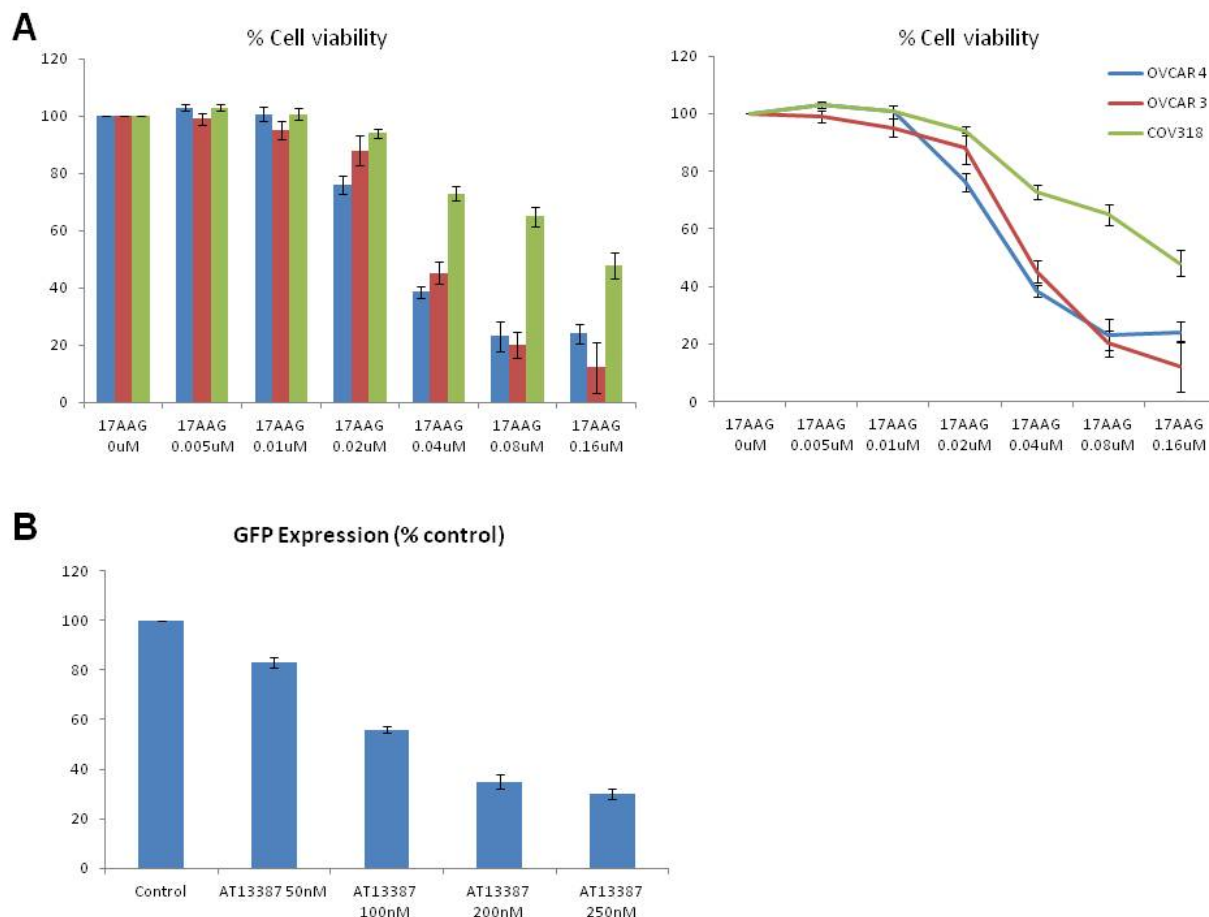


Figure 1. **A)** Activity of 17-AAG in OVCAR3, COV318 CCNE1 amplified and OVCAR4 CCNE1 overexpressing ovarian cancer lines. **B)** HSP90 inhibitor AT13387 suppresses HR as assessed by the Direct Repeat-GFP (DR-GFP) reporter assay.

The inferior activity may be related to inherent resistance to 17-AAG which may relate to mutations in HSP90 that affects the interaction with 17-AAG or overexpression of drug efflux pump MDR1 or elevated baseline HSP70 levels. We are currently assessing whether these mechanisms may contribute to this differential sensitivity of CCNE1-amplified lines to 17-AAG.

2) HSP90-inhibition downregulates homologous recombination (HR) DNA repair

One of our hypothesis of how HSP90 may have activity against CCNE1 amplified cells is based on the fact that CCNE1 amplified cells are hyperdependent on an intact HR (2). Therefore, by downregulating HR, HSP90 inhibitors may induce lethality in CCNE1 amplified cells. In this regard, during this funding period, we assessed whether the HSP90 inhibitor AT13387 suppresses HR using the Direct Repeat-GFP (DR-GFP) reporter assay (3).

In this assay, there is measurement of HR-mediated repair of an I-SceI induced site specific DSB. Specifically, 0.1×10^6 U2OS cells carrying DR-GFP reporter were plated on a 12-well plate overnight, treated with 17 AAG at indicated concentrations for 24 hrs,

and transfected with 500 ng of I-SceI expression plasmid or control vector using Lipofectamine 2000. After 48 h, GFP-positive cells were assayed by FACSscan.

As shown in Figure 1B, increasing concentrations of AT13387 reduced the efficacy of HR DNA repair by approximately 70%, down to plateau of 30%. This is also being confirmed using the RAD51 foci formation after ionizing radiation (IR) assay.

3) The HSP90-inhibitor AT13387 synergizes with DNA damage inducing agents such as PARP-inhibitors against CCNE1-amplified cell lines

One of the key goals of Aim 1 was to also assess the activity of HSP90 inhibitors in combination with platinum and PARP inhibitors in CCNE1-amplified cell lines. During this funding period we assessed the combination of the novel PARP inhibitor BMN673 (talazoparib) (4) in combination with the HSP90 inhibitor AT13387.

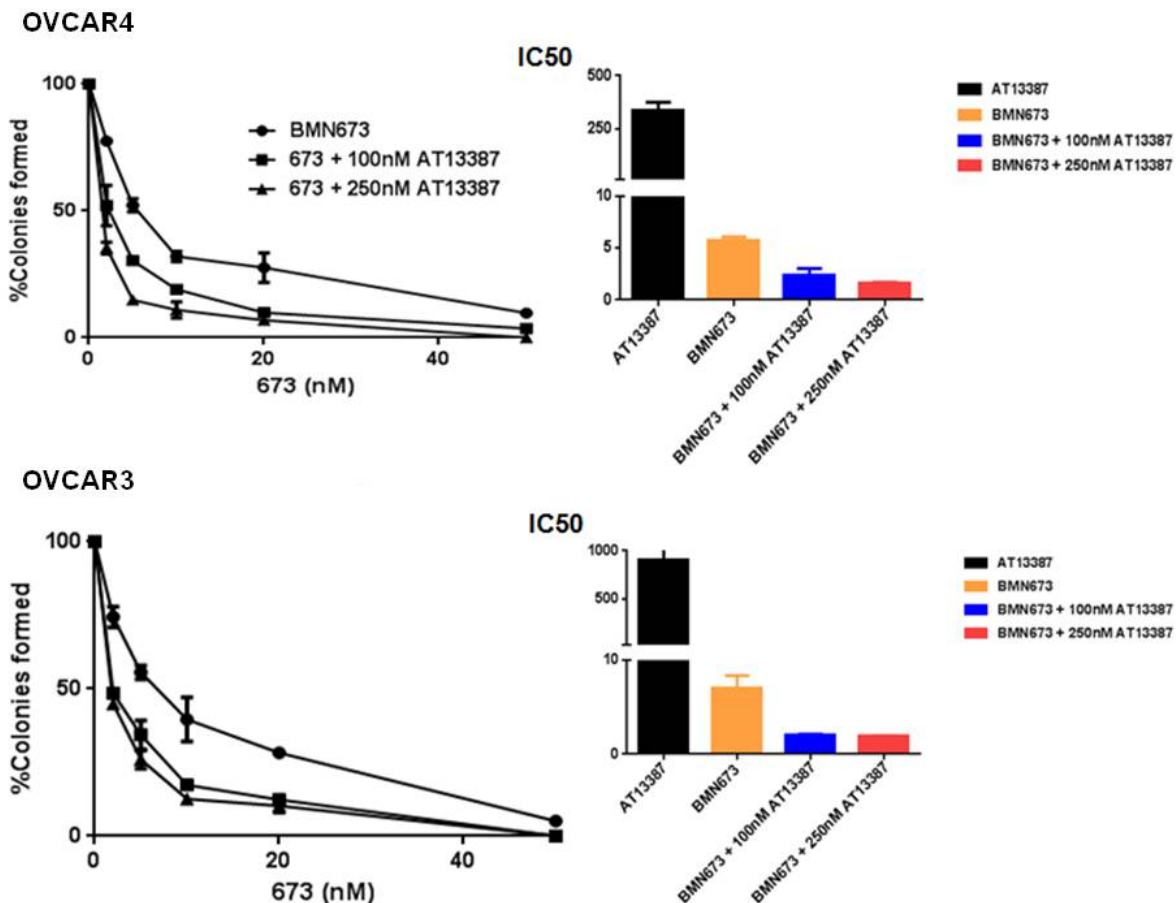


Figure 2. In vitro synergism between HSP90 inhibitor AT13387 and the PARP inhibitor BMN673 in OVCAR4 and OVCAR3.

We have now assessed the combination talazoparib and AT13387 in OVCAR4 and OVCAR3 which exhibit CCNE1 overexpression and amplification respectively using colony formation assay. Cells were treated with the indicated (Figure 2) BMN673 and

AT13387 concentrations. As shown in Figure 2, in both cell lines, addition of small concentrations of AT13387 enhanced the cytotoxicity of BMN673, indicating synergism between BMN673 and AT13387. We plan to assess other PARPis as well, including olaparib.

For AIM 2:

The retinoblastoma protein (RB) is a tumor suppressor that functions downstream of cyclin E1 (encoded by the *CCNE1* gene) to regulate cell cycle, apoptosis and differentiation through its direct binding to and inhibition of the E2F transcription factor (5, 6). Disruption of RB and E2F interaction by viral oncogenic proteins such as HPV-E7 leads to neoplastic transformation (7). HPV-E7 inhibits RB function through a conserved LxCxE motif for high affinity RB binding (7, 8). Although RB pathway including its upstream regulator cyclin E is often deregulated in EOC (9), genetic alterations of the RB gene itself are relative rare in EOC (10-12). Notably, RB physically interacts with FOXM1 (13, 14), a transcription factor with oncogenic activity in EOC (9). Interestingly, the FOXM1 transcriptional network is significantly upregulated in EOC as well (9). Thus, we sought to determine whether the interaction between RB and FOXM1 can be targeted in EOC. Since RB functions downstream of cyclin E, we expect that this approach will be especially effective in *CCNE1* amplified EOCs.

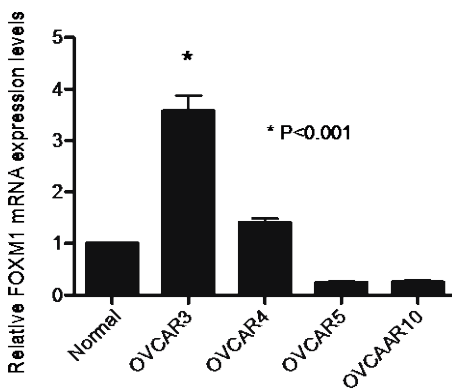


Figure 3. Expression of FOXM1 mRNA in a panel of EOC cell lines and normal fallopian tube epithelial cells determined by qRT-PCR.

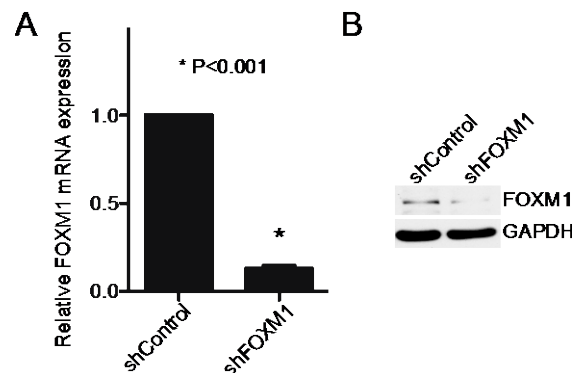


Figure 4. shRNA mediated knockdown of FOXM1 in OVCAR3 cells. Expression of FOXM1 in OVCAR3 expressing shFOXM1 or controls was determined by qRT-PCR (A) or immunoblotting (B).

Toward this goal, we first examined the expression of *FOXM1* mRNA in a panel of EOC cell lines by qRT-PCR. We used normal fallopian tube epithelial cells as a control. Notably, OVCAR3, a cell lines with known *CCNE1* amplification showed the highest levels of FOXM1 expression (**Figure 3**). Thus, we used OVCAR3 cells to perform the subsequent functional studies. To determine the role of FOXM1 in OVCAR3 cells, we developed a shRNA targeting the human FOXM1 gene. We validated the knockdown efficiency fo shFOXM1 by both qRT-PCR and immunoblotting (**Figure 4**). Supporting the notion FOXM1 is required for the proliferation of *CCNE1* amplified EOC cells. FOXM1 knockdown significantly suppressed the growth of OVCAR3 cells as determined by both cell growth curve and colony formation assays (**Figure 5**).

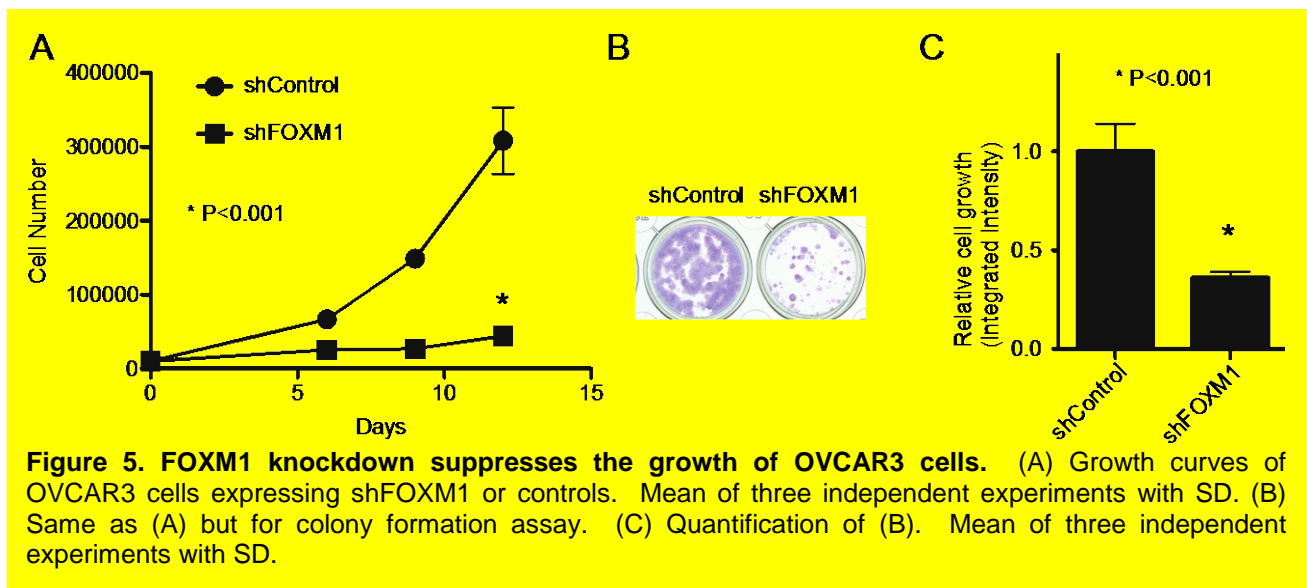


Figure 5. FOXM1 knockdown suppresses the growth of OVCAR3 cells. (A) Growth curves of OVCAR3 cells expressing shFOXM1 or controls. Mean of three independent experiments with SD. (B) Same as (A) but for colony formation assay. (C) Quantification of (B). Mean of three independent experiments with SD.

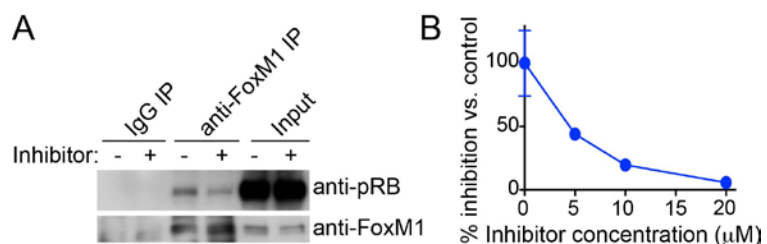


Figure 6. An inhibitor of FOXM1 and RB interaction that inhibits the growth of CCNE1 amplified NIH-OVCAR3 EOC cells. A) CCNE1 amplified NIH -OVCAR3 cells were treated with 5 mM inhibitor 478726 or control inactive compound 77333 for 48 h. The indicated cells were used for co-immunoprecipitation assay using an anti-FOXM1 antibody or a control IgG. The IPed product were examined by immunoblotting using the indicated antibodies. B) Same as A) but examined cell growth using the indicated concentration of inhibitor or control compound for 72 hours. Curves represent cell numbers normalized to control treatment.

The binding between FOXM1 and RB depends upon a LxCxE motif on FOXM1 (13, 14). Notably, a class of thiadiazolidinedione compounds have previously been identified that disrupt the LxCxE motif mediated interaction between HPV-E7 and RB (15). These compounds are selectively cytotoxic in HPV-positive cells *in vitro* and *in vivo* in mouse models (15). The observed effects correlate with its ability to suppress the disruption of RB/E2F complex by HPV-E7. This leads to a restoration of

RB/E2F interaction and suppression of E2F dependent oncogenic activity. Since FOXM1's interaction with RB is also dependent upon the LxCxE motif (7, 8), we examined the effects of the RB/HPV-E7 disruption compound 478166 (or inhibitor 478726) on the interaction between FOXM1 and RB in EOC cells. In CCNE1 amplified NIH-OVCAR3 cells (16), the interaction between FOXM1 and RB is readily detectable (Figure 6A). Co-immunoprecipitation analysis revealed that the interaction between

FOXM1 and RB is substantially suppressed by inhibitor 478726 (**Figure 6A**). Since the disruption of interaction between HPV-E7 and RB by the inhibitor leads to cell growth arrest in HPV positive human cancer cells (15), we examined whether disruption of FOXM1 and RB also inhibits the growth of *CCNE1* amplified NIH-OVCAR3 cells. Indeed, we observed a dose dependent suppression of cell growth by the inhibitor 478726 in these cells (**Figure 6B**). In summary, our preliminary data identified a small molecule inhibitor that can disrupt the interaction between FOXM1 and RB, which correlates with a dose-dependent growth inhibition in *CCNE1* amplified EOC cells.

In summary, we have demonstrated in Aim 2:

- 1) FOXM1 is necessary for the proliferation of *CCNE1* amplified epithelial ovarian cancer cells.
- 2) FOXM1 interacts with Rb in *CCNE1* amplified epithelial ovarian cancer cells.
- 3) Characterized small molecule inhibitor that disrupts the interaction between FOXM1 and Rb in *CCNE1* amplified epithelial ovarian cancer cells.

For AIM 3:

During the first year of the award we were able to achieve the following goals:

1) Certain miRNAs including miR-1255b, miR-148b*, and miR-193b* inhibit HR DNA repair

As proposed in Aim 3, we evaluated whether certain miRNAs may inhibit HR repair in *CCNE1* amplified cell lines. To achieve this we assessed the effects of miRNA mimics for miR-1255b, miR-148b*, and miR-193b* and miR-182 in *CCNE1* amplified OVCAR3 cells using the RAD51 foci formation after ionizing radiation (IR) assay. Specifically, OVCAR3 cells were transfected with control miRNA mimic and the indicated (Figure 7) miRNA mimics stained for RAD51 (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue) 6 h after exposure to IR. The images were captured by fluorescence microscopy and RAD51 focus-positive cells (with > 5 foci) were quantified by comparing 100 cells.

As shown in Figure 7, treatment with miRNA mimics for miR-1255b, miR-148b*, and miR-193b* and miR-182, significantly reduced RAD51 foci formation after IR compared to control miRNA mimic, suggesting that these miRNA mimics can indeed inhibit HR repair in these *CCNE1* amplified cells.

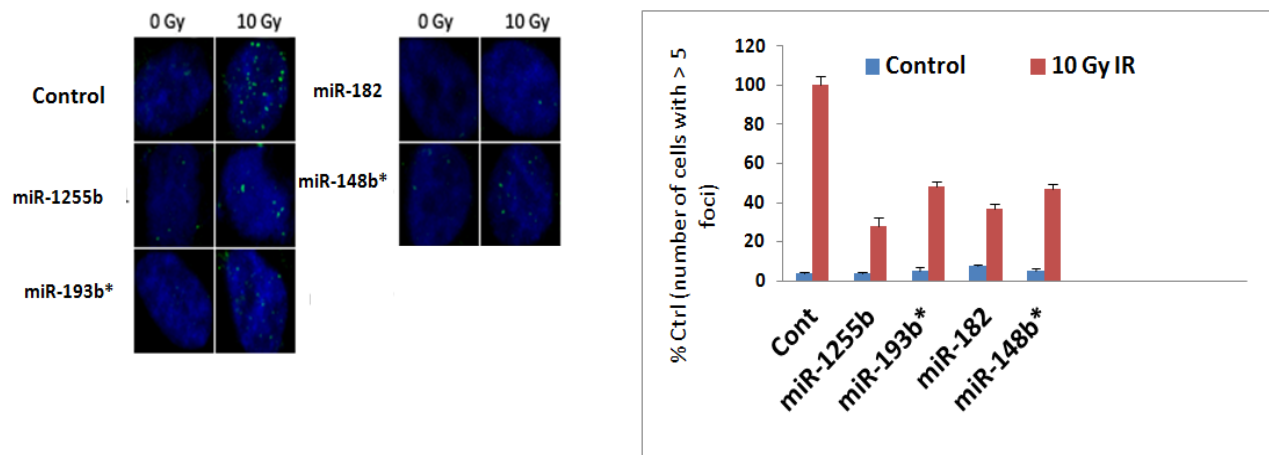


Figure 7. miRNA mimics for miR-1255b, miR-148b*, and miR-193b* and miR-182 inhibit HR repair in CCNE1 amplified OVCAR3 cells

2) These miRNAs synergize with platinum and PARP-inhibitors against CCNE1-amplified cell lines, that is expression of these miRNAs sensitizes CCNE1-amplified cells to platinum and PARP-inhibitors.

Another important goal of Aim 3, was to assess whether these miRNAs may synergize with platinum and PARPis in CCNE1-amplified cells.

To achieve this, luminescence based viability assay was performed in OVCAR3 and OVCAR4 ovarian cells. Cells were transfected with control miRNA, miRNA mimics for miR-1255b, miR-148b* and miR-193b* or BRCA1 siRNA (positive control). All cells were concomitantly treated with 1 μ M PARP inhibitor AZD2281 (Olaparib) and increasing concentrations of cisplatin for 5-6 days before ATP quantification.

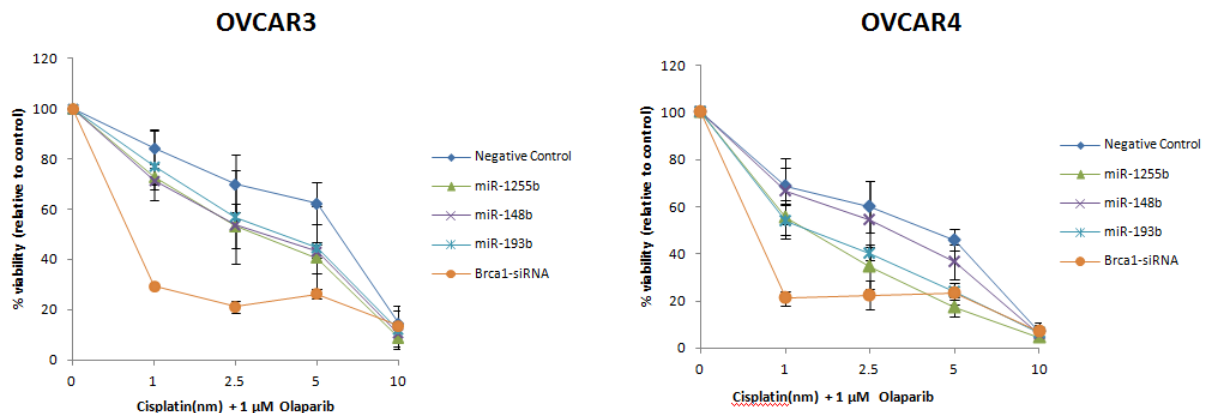


Figure 8. miRNA mimics for miR-1255b, miR-148b* and miR-193b* enhance cytotoxicity to cisplatin and olaparib.

As shown in Figure 8, miRNA mimics for miR-1255b, miR-148b* and miR-193b* enhanced sensitivity to cisplatin and olaparib, more than the negative control and less than the positive control BRCA1 siRNA. These findings, together with our finding that these miRNA mimics inhibit HR in CCNE1 amplified cells, support our hypothesis and suggest a novel strategy for targeting CCNE1 amplified tumors. In the next funding period, we plan to perform studies of miRNA mimics with additional PARP-inhibitors and platinum agents, either alone or in combination, in CCNE1-amplified ovarian cancer cells.

What opportunities for training and professional development did the project provide?

“Nothing to Report.”

How were the results disseminated to communities of interest?

“Nothing to Report.”

What do you plan to do during the next reporting period to accomplish the goals and objectives?

In the next reporting period:

For Aim 1, we plan to perform synergism studies of HSP90-inhibitors with additional PARP-inhibitors and with platinum agents in CCNE1-amplified ovarian cancer cells. We also intend to initiate tolerability studies of these agents in patient-derived xenografts (PDX) models of CCNE1-amplified ovarian cancer.

For Aim 2, we plan to accomplish whether and how the inhibitor of FOXM1 and RB interaction affects the growth of CCNE1-amplified epithelial ovarian cancer cells.

For Aim 3, we plan to perform synergism studies of miRNA mimics with additional PARP-inhibitors and platinum agents in CCNE1-amplified ovarian cancer cells. We also intend to evaluate possible targets of these miRNAs which explain their action of suppressing HR DNA repair.

4. IMPACT:

“Nothing to Report.”

What was the impact on the development of the principal discipline(s) of the project?

“Nothing to Report.”

What was the impact on other disciplines?

"Nothing to Report."

What was the impact on technology transfer?

"Nothing to Report."

What was the impact on society beyond science and technology?

"Nothing to Report."

5. CHANGES/PROBLEMS:

"Nothing to Report."

Changes in approach and reasons for change

"Nothing to Report."

Actual or anticipated problems or delays and actions or plans to resolve them

"Nothing to Report."

Changes that had a significant impact on expenditures

"Nothing to Report."

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

"Nothing to Report."

6. PRODUCTS:

- Publications, conference papers, and presentations**

1. Zhu H, Bengsch F, Svoronons N, Rutkowski MR, Bitler BG, Allegrezza MJ, Yokoyama Y, Bradner JE, Conejo-Garcia JR, Zhang R. BET bromodomain

- inhibition promotes anti-tumor immunity by suppressing PD-L1 expression. **Cell Reports**, 16:2829-37, 2016.
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 3. **Konstantinopoulos PA**, Ceccaldi R, Shapiro GI, D'Andrea AD. Homologous Recombination Deficiency: Exploiting the Fundamental Vulnerability of Ovarian Cancer. (2015) **Cancer Discovery** 5(11):1137-54.
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Books or other non-periodical, one-time publications.

"Nothing to Report."

Other publications, conference papers, and presentations.

"Nothing to Report."

- **Website(s) or other Internet site(s)**

"Nothing to Report."

- **Technologies or techniques**

"Nothing to Report."

- **Inventions, patent applications, and/or licenses**

"Nothing to Report."

- **Other Products**

"Nothing to Report."

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	<i>Panagiotis Konstantinopoulos</i>
Project Role:	<i>Principal Investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Konstantinopoulos has performed work related to the Aim 1 of this project.</i>
Funding Support:	<i>DOD Ovarian Cancer Research Program – Ovarian cancer Academy Award Ovarian Cancer Research Foundation The Honorable Tina Brozman Foundation for Ovarian Cancer Research Stand Up to Cancer</i>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

“Nothing to Report.”

What other organizations were involved as partners?

“Nothing to Report.”

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

We here include the references relevant to this report.

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1. Domcke S, Sinha R, Levine DA, Sander C, Schultz N. Evaluating cell lines as tumour models by comparison of genomic profiles. *Nat Commun.* 2013;4:2126.
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